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Atty. Docket No.: P64765US0
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Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Transmitted herewith for filing is a PROVISIONAL APPLICATION of **Peet KASK** residing at **Uus 5-3, Harku EE 3051, ESTONIA** for **FLUORESCENCE INTENSITY MULTIPLE DISTRIBUTION ANALYSIS A METHOD OF ANALYSING FLUORESCENCE**. The application comprises a 8 -page specification.

Accompanying this application for filing is:

_X_ Filing Fee: __ Small Entity, $75.00 _X_ Large Entity, $150.00

Check No. 46277, in the amount of $ 150.00, is enclosed to cover the Filing Fee. The Commissioner is hereby authorized to charge payment of any fees set forth in §§1.16 or 1.17 during the pendency of this application, or credit any overpayment, to Deposit Account No. 06-1358. A duplicate copy of this sheet is enclosed.

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Respectfully submitted,

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# Fluorescence Intensity Multiple Distribution Analysis
## A Method of Analysing Fluorescence


## ABSTRACT

FCS (Fluorescence Correlation Spectroscopy) and FIDA (Fluorescence Intensity Distribution Analysis) are two distinct methods of analyzing fluorescence fluctuations caused by the diffusion of fluorescent particles through an open volume. From FCS the diffusion time and from FIDA the molecular brightness of different species can be deduced. Here we introduce a new method (FIMDA, Fluorescence Intensity Multiple Distribution Analysis) which combines both, FCS and FIDA. Within one measurement, three parameters are determined for each species: the diffusion time $\tau$, the molecular brightness value q and the concentration c. Like in FIDA, the analysis is applied to photon count number distributions. In FIMDA, however, a set of count number distributions is used, with each distribution corresponding to a different length of counting time interval. Since the values for all three parameters are attributed to each species, FIMDA is a more powerful tool of analysis than applying FCS and FIDA separately. The statistical accuracy of each extracted parameter in FIMDA is comparable to that in FCS or FIDA, in some cases even better. We demonstrate this new method to be a sensitive and selective tool in determining the binding constant of a protein-ligand interaction.


## THEORY

The central part in FIDA (1), (2), is the collection of photon count numbers, recorded in time intervals of fixed duration (time windows) and using this information to build up a count number histogram. A theoretical probability distribution of photon count numbers is fitted against the obtained histogram, yielding specific brightness values as well as concentrations for all different species in the sample. The historic predecessor of FIDA is FCS, which distinguishes different species on the basis of their characteristic diffusion times $\tau$, by analyzing the second order autocorrelation function of light intensity. Parameters which can be determined from FCS (in addition to diffusion times $\tau$) are not, however, concentrations $c$ and specific brightness values $q$ of different species separately, but products of the form $cq^2$.

The key in FIMDA is to analyze a set of distributions, which is sensitive to the translational diffusion of particles. FCS detects the dynamics of particles, because it compares the instantaneous intensities at time intervals separated by a certain delay. In

order to make the distribution of photon count numbers sensitive to the temporal evolution of intensity one may alternatively choose to build a set of photon count number histograms corresponding to different time windows. The choice of the time windows should span a range comparable to the delay values used in FCS.

In FIDA, a convenient representation of a photon count number distribution P(n) is its generating function, defined as

$$R_{P(n)}(\xi) = \sum_n \xi^n P(n). \quad (1)$$

The simple theory of FIDA assumes (i) that molecules are immobile during the counting time interval, and (ii) that the light flux from a molecule can be expressed as a product of a spatial brightness function $B(\mathbf{r})$ (this is a function of spatial coordinates of the molecule characterizing the equipment) and a specific brightness $q$ (characterizing a certain molecular species). Under these two assumptions, the distribution of the number of photon counts, emitted by molecules from a volume element $dV$, is double Poissonian and the corresponding generating function reads

$$R_{P(n)}(\xi) = \exp\left[cdV\left(e^{(\xi-1)qB(\mathbf{r})T}-1\right)\right], \quad (2)$$

where $\xi$ is the complex argument of the generating function, $c$ is the concentration of molecules, and $T$ is the width of the counting time interval. The representation we use is convenient, because contributions from independent sources, like different volume elements or species, are
combined by simple multiplication of the contributing generating functions.
The generating function of $P(n)$ for a single species is

$$R_{P(n)}(\xi) = \exp\left[c\int\left(e^{(\xi-1)qB(\mathbf{r})T}-1\right)dV\right], \quad (3)$$

while accounting for multiple species simply yields

$$R_{P(n)}(\xi) = \exp\left[\sum_i c_i \int\left(e^{(\xi-1)q_i B(\mathbf{r})T}-1\right)dV\right]. \quad (4)$$

The integral on the right hand side of Eq. 4 is calculated numerically. In order to define the confocal volume and adjust the instrument, normalization conditions (for brightness and volume) are introduced. So far, we have described a simple version of FIDA. For the purposes of FIMDA, we have to abandon the assumption that molecules are immobile during the counting interval. Surprisingly, we will not abandon Eq. 2, but we will redefine the meaning of some variables instead. $\mathbf{r}$ is still a variable related to the spatial brightness profile, but now it characterizes the path of the molecule rather than its position. $B$ is the spatial brightness averaged over the path rather than determined at a fixed position of the molecule. $V$ is not the volume in space but $dV/dr$ still expresses the

probability that a molecule has a given value of **r**. If we would keep the original meaning of *c* and *q*, we would have to develop a theory predicting how the confocal volume depends on the counting time interval *T*. However, we have chosen another approach. We kept the normalization conditions and even found it possible to apply *one* single shape of the apparent brightness function to a set of *different* time windows. The consequence of this selection is that in Eqs. 2-4 *c* is an apparent concentration ($c_{app}$) and *q* is an apparent brightness ($q_{app}$) which both depend on the width of the counting time interval *T*.

Applying some algebra which basically relates the second factorial cumulant of the count number distribution to the autocorrelation function G(t) we arrive at the apparent brightness and apparent concentration:

$$q_{app}(T) = q\Gamma(T), \qquad (5) \qquad c_{app}(T) = \frac{c}{\Gamma(T)}, \qquad (6)$$

with the abbreviation

$$\Gamma(T) = \frac{1}{cq^2T^2}\int_0^T dt_1 \int_0^T dt_2 G(t_2 - t_1). \qquad (7)$$

**Experimental set-up**

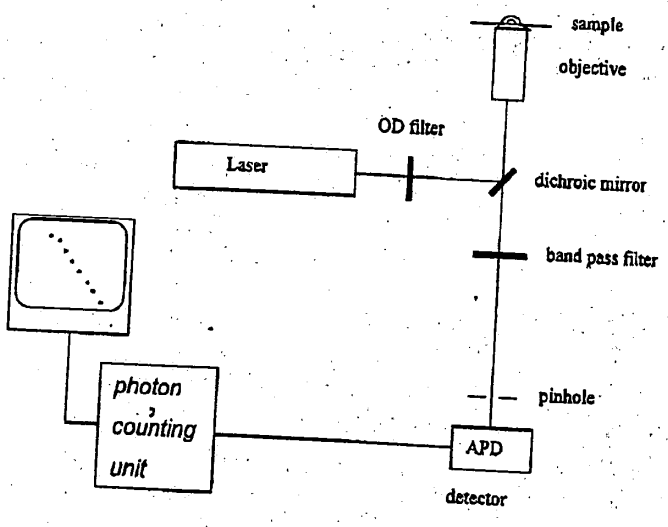

Figure 1: A beam from a continuous wave laser is directed to a microscope objective by a dichroic mirror. The fluorescence light is collected by the same objective and passes a confocal pinhole, before it is detected by a silicon photon counting module.

## RESULTS

Fig. 1 shows the experimental set-up used in this study. As an example for a typical measurement Fig. 2A shows 10 count number distributions with time windows 40, 60, 120, 200, 400, 600, 800, 1200, 1600, 2000 µs from a 0.8 nM Cy 5 solution. Fig. 2B shows the calculated apparent molecular brightness of the dye as a function of counting time interval (time window), evaluated by FIDA. Since the diffusion time of the dye is long (compared to 40 µs) it spends only a short time in the confocal volume and its apparent brightness decreases. From this information the diffusion time can be calculated from a global fit.

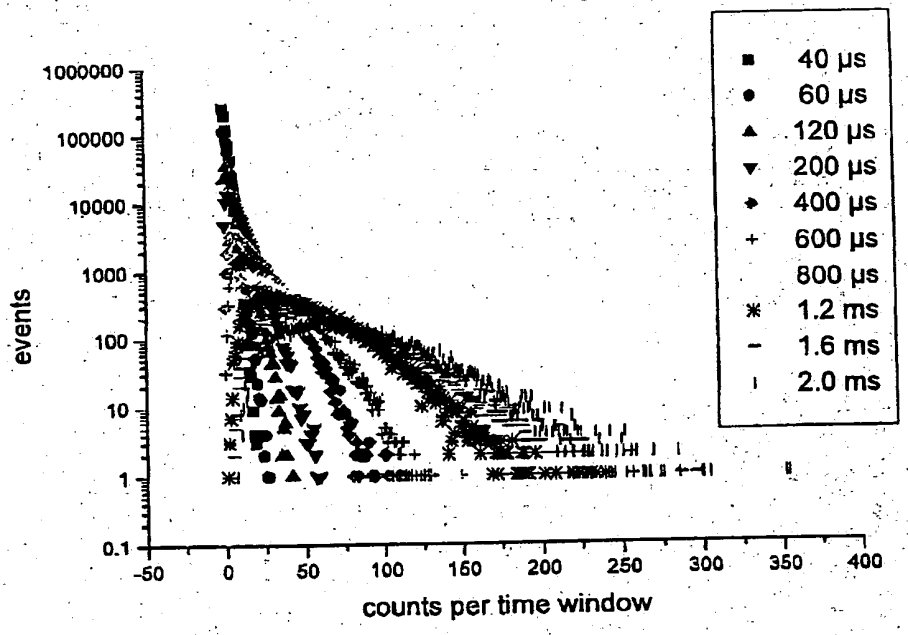


Figure 2A: Ten different count number distributions, measured simultaneously on a 0.8 nM Cy5 solution.

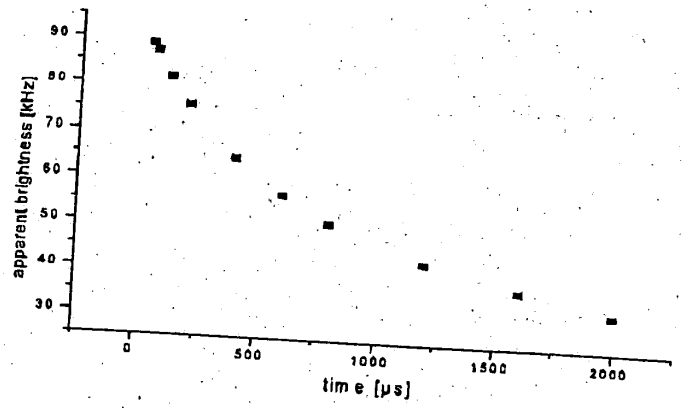


Figure 2B: Apparent molecular brightness as a function of time window, as determined from a 0.8 nM Cy 5 solution.

**MEASUREMENTS on a biochemical system**

We tested the viability of this new method by measurements characterizing a ligand-protein interaction. The minimal peptide sequence pTyr-Val-Asn (originating from tyrosine kinase growth factor receptors) binds to the SH2-domain of the adapter protein Grb2 . We synthesized the conjugate pTyr-Val-Asn-Val-Lys(Cy5) as binding partner for SH2. All measurements were performed under identical conditions (e.g. same buffer: sterile filtered water, 50 mM Na-phosphate buffer pH 7.8, 50 mM NaCl and 0.05 % Pluronic; T=20°C). To obtain the $K_D$ value for the ligand-protein interaction a titration experiment was carried out. The pTyr-Val-Asn-Val-Lys(Cy5) concentration was kept constant at 0.8 nM, while SH2 was subject to titration (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, and 130 µM). Each sample was measured 30 times keeping the acquisition time at 30 seconds per measurement. FCS, FIDA and FIMDA data were collected simultaneously, in order to compare all three methods. Table 1 summarizes the results. It is obvious that FIMDA is able to resolve both, the molecular brightness q and the diffusion time $\tau$ of both species (ligand and complex). Therefore FIMDA is a more versatile tool than FCS or FIDA and will save assay development time. Comparing the standard deviations of all methods in determining q and $\tau$ FIMDA is better or at least as good as FCS or FIDA. FCS, FIDA and FIMDA data were collected simultaneously, in order to compare all three methods. Table 1 summarizes the results. ~~It is obvious that~~ FIMDA is able to resolve both, the molecular brightness q and the diffusion time $\tau$ of both species (ligand and complex). Therefore FIMDA is a more versatile tool than FCS or FIDA and will save assay development time. Comparing the standard deviations of all methods in determining q and $\tau$ FIMDA is better or at least as good as FCS or FIDA.

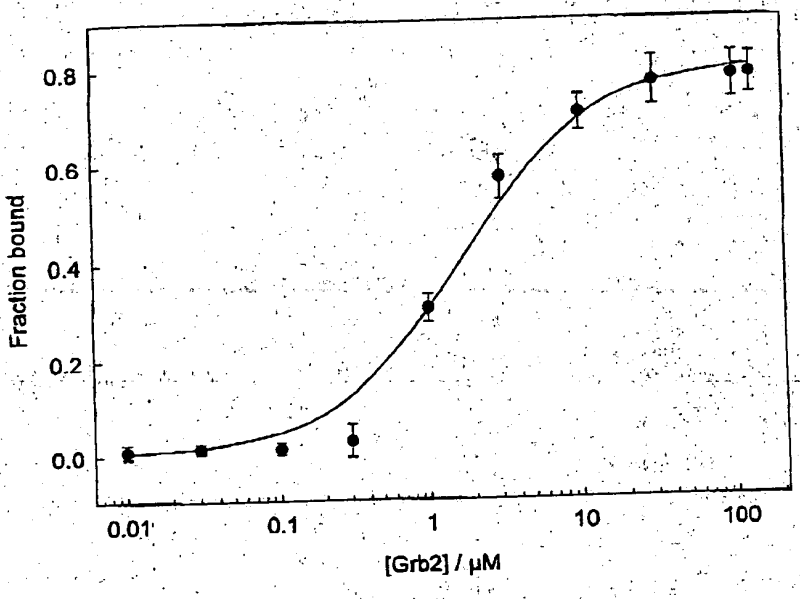


Fig. 3: Binding curve calculated from ten different count number distributions taken from a phosphopeptide - protein interaction

Table 1: Comparison of FIMDA with FCS and FIDA with respect to the determination of q, $\tau$ and $K_D$

| | $q_1$ [kHz] | $q_2$ [kHz] | $\tau_1$ [ms] | $\tau_2$ [ms] | $K_D$ [μM] |
|---|---|---|---|---|---|
| FIMDA | 31.7±0.32 | 39.6±0.9 | 0.402±0.006 | 1.01±0.08 | 1.68± 0.27 |
| FCS | | | 0.402±0.013 | 0.867±0.057 | 2.26±0.28 |
| FIDA | 32.1±0.31 | 43.3±2.3 | | | 1.70±0.29 |

**Summary**

- **Because two readout parameters are recorded in one measurement with one detector, FIMDA is an efficient and reliable method of analysis.**
- **The introduction of the generating function facilitates data evaluation and makes the method a fast analytic tool.**
- **FIMDA is more universal applicable to biochemical assays than FCS and FIMDA applied separately, because each assay component can be distinguished by means of both, diffusion time and brightness.**
- **The statistical accuracy of determining the diffusion time in FIMDA is better or at least as good as in FCS.**

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